Biofuel Cells

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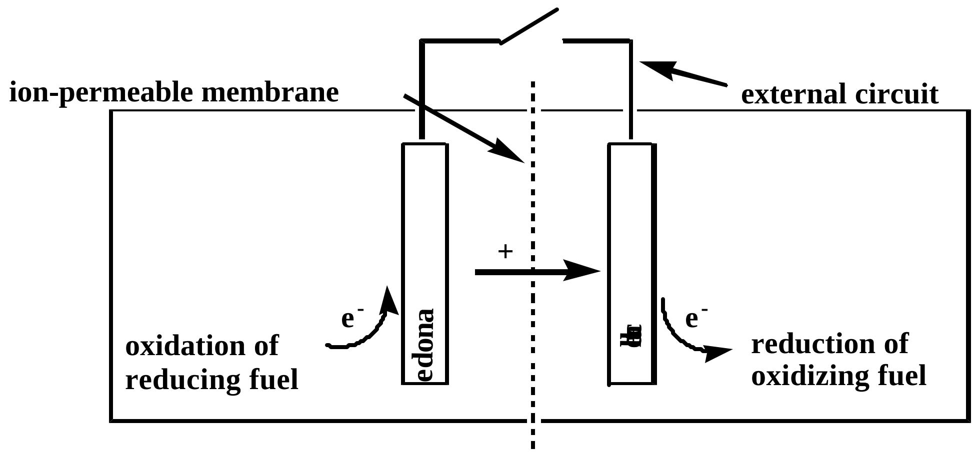
Overall goal:

- Develop a fuel cell that uses biocatalysts in both the anode and cathode compartments.

Specific goals:

- identify, isolate and characterize biocatalysts for specific reactions (e.g., oxidation of dihydrogen, methanol, or glucose; reduction of dioxygen)
- develop methodology for interfacing biocatalysts with electrode surfaces (e.g., mediation, immobilization)
- improve the catalytic characteristics of the biocatalysts using molecular biology and chemistry (e.g., pH and temperature stability)

A fuel cell converts chemical energy directly into electrical energy



Why are fuel cells interesting?

- efficient (not limited by the Carnot cycle)
- simplicity in design
- non-polluting (noise, emissions)

What are the important parameters?

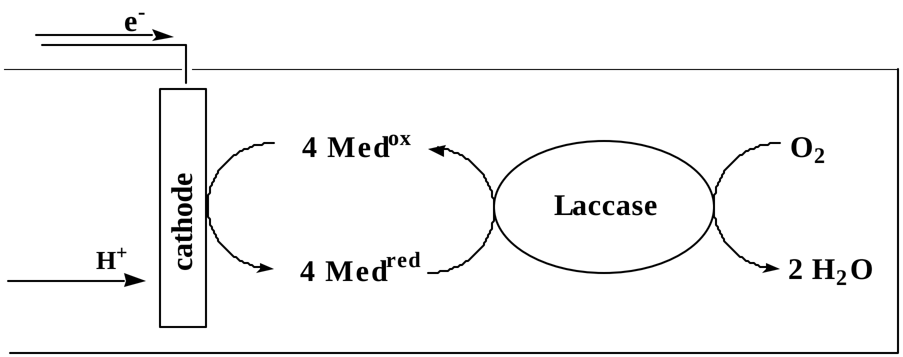
- power density = watts per area of electrocatalyst
- watt = V_{cell} • amps
- $V_{cell} = V_{cathode} - V_{anode} - IR$ (glucose/dioxygen fuel cell, $V_{cell} \sim 1.1$ V)
- amps = C/s (rate of catalysis, electron transfer, mass transport)

Prototype H_2/O_2 biofuel cell

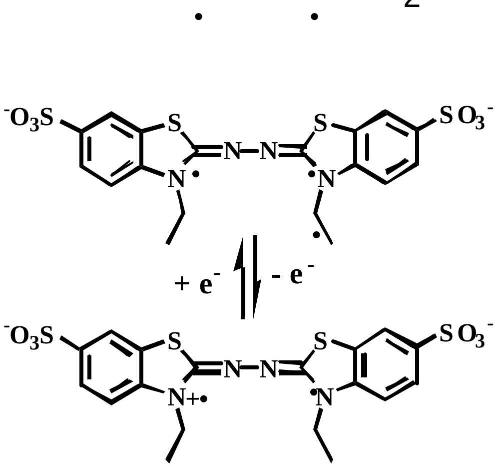
Fuel Cell: two glass cells containing 0.2 M acetate buffer (pH 4.0) separated by a Nafion membrane, operated at ambient temperature and pressure.

Anode compartment: Pt black, dissolved H_2

Cathode compartment: carbon, laccase, ABTS, dissolved O_2

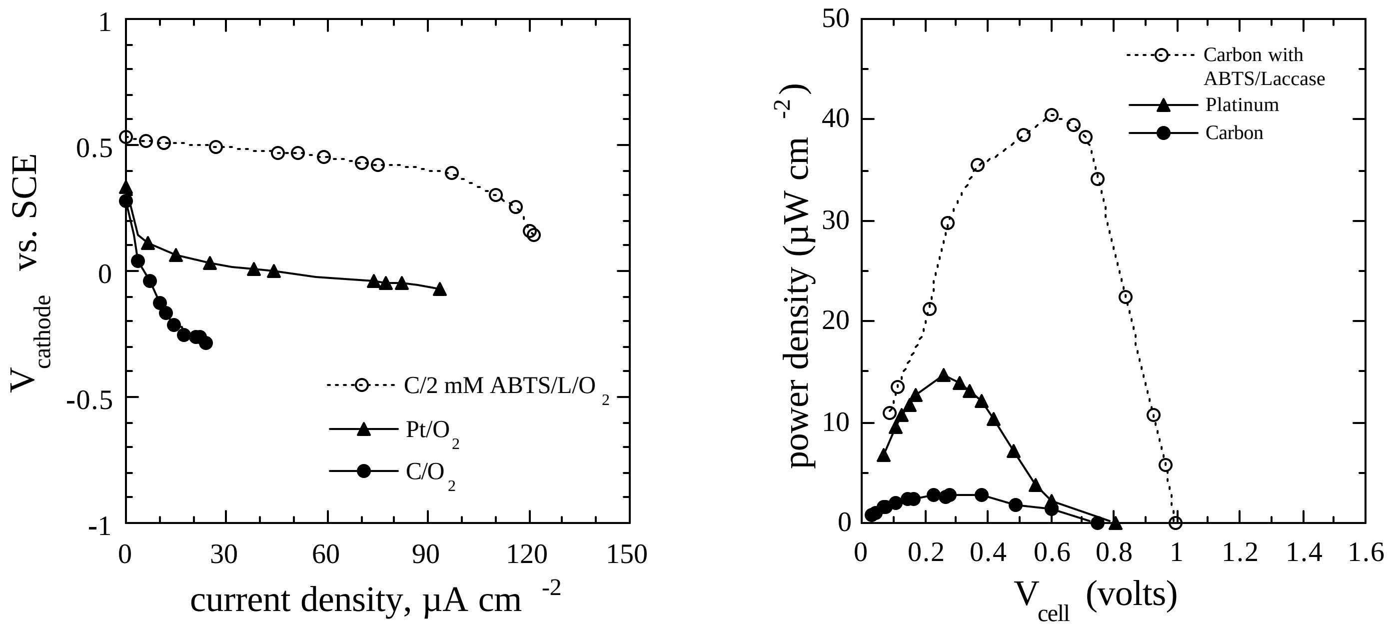


"reformer" cathode (mediated electron transfer)



$Med^{ox/red}$ = ABTS

Prototype H_2/O_2 biofuel cell



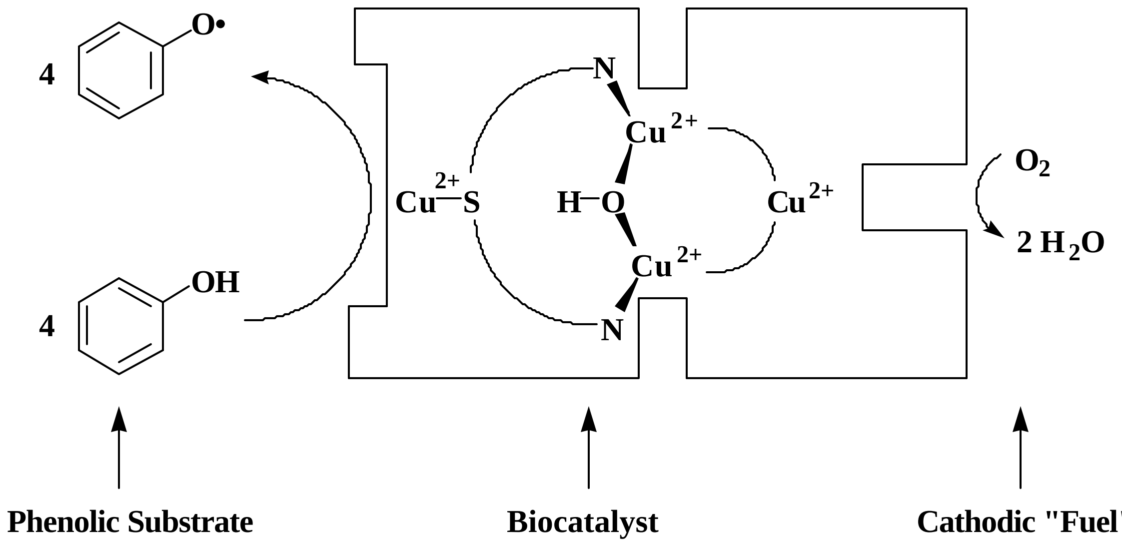
- Open circuit voltage of biocathode is 0.53 V vs. SCE
- Current-voltage curve measured with external loads of 50 k - 100
- Concentration overpotential at current densities > 100 µA cm^{-2}
- Maximum power density at 1 k load is 42 µW cm^{-2} at 0.61 V

Limitations of technology used in the prototype H_2/O_2 biofuel cell

- Biocatalyst (e.g., commercial laccase, activity, inhibition, gene shuffling and heterologous expression)

- Biofuel cell design (e.g., glucose/O_2)
  - Concentration overpotentials
  - Membrane
  - Mediator

Active site of Laccase



Isolated from fungi, plants, bacteria; 50-70 kDa; isoelectric point 3-7; 5-50% glycosylation; three distinct coordination sites for Cu (4/subunit)

- one type 1 Cu (λ_{max} = 615 nm, E°' = 0.35-0.79 V)
- one type 2 Cu (EPR tetragonal coordination, E°' = 0.37 vs. NHE)
- two type 3 Cu (Cu pair, λ_{max} = 330 nm, E°'= 0.36-.78 V, EPR silent)

low specificity for reducing substrate

high specificity for dioxygen substrate

Commercial laccases from Sigma

- Laccase (*Rhus vernificera*) purified from natural host (i.e., plant)
- Catalytic constant with syringaldazine (pH 6.5, 30 °C) is in the range of 50 U mg^{-1} solid (~50 s^{-1}), essentially inactive with ABTS due to “low potential” copper sites
- $0.30 per mg

- Laccase (*Pyricularia oryzae*) purified from natural host (i.e., fungus)
- Catalytic constant with syringaldazine (pH 6.5, 30 °C) is in the range of 144-389 U mg^{-1} solid (~156-421 s^{-1})
- Maximum activity at pH 3.0
- ~25% of maximum activity retained at pH 7
- no longer available

Commercial laccase from SynectiQ

- Laccase (*Coriolus sp*.) purified from natural host (i.e., mushrooms)
- Reported catalytic constants for most substrates (e.g., ABTS, syringaldazine, $K_4[Fe(CN)_6]$, Fe^{2+} ions) are in the range of ~100-500 s^{-1}
- Maximum activity at pH 3.5-4.5
- 1-10% of maximum activity at pH 7 (i.e., 1-50 s^{-1})
- $50 per mg

Activity vs. Structure of Substrate

4-substituents on 2-methoxyphenol	**MW**	**K_m, mM**	**k_{cat}, min^{-1}**
4-$CH_2CO_2^-$	58	0.10±0.02	2200±100
4-$CH_2CONH(CH_2)_6NH_3^+$	158	0.20±0.02	1700±100
4-$CH_2CONH(C_6H_6)NCO_2CH_3$	191	0.27±0.03	2100±100
4-CH_2CONH-lysozyme	14000	0.40±0.06	1400±100
4-$CH_2CONH(CH_2)_6$NHCO-lysozyme	14200	0.33±0.06	1400±100

Consistent with structural data, kinetic data indicates a shallow substrate pocket (10 Å deep, 15 Å in diameter) in that can accommodate substrates with a variety of shapes

Redox potential of the Cu-sites

	$E°$, V vs. NHE, pH 7		
Laccase			
Dioxygen/water	0.79 (1.0 @ pH 3.5)		
	T1	**T2**	**T3**
Trametes versicolor	0.79	---	0.78
Botrytis cinerea (BcL)	0.78	---	---
Trametes villosa (TvL)	0.77	---	---
Pycnoporus cinnabarinus (PcL)	0.75	---	---
Rhizoctonia solani (RsL)	0.71	---	---
Coprinus cinereus (CcL)	0.55	---	---
Scytalidium thermophilum (StL)	0.51	---	---
Myceliophthora thermophila (MtL)	0.47	---	---
Rhus vernicifera (RvL)	0.45	0.37	0.43
Myrothecium bilirubin oxidase (MvBO)	*0.49*	---	---
Human serum ceruloplasmin	*0.49*	---	---
Ascorbate oxidase	*0.35*	---	*0.36*

Interaction of Anions with Laccase

Laccase	*F^-	*Cl^-	*Br^-
Trametes villosa (TvL)	0.02	40	200
Rhizoctonia solani (RsL)	0.02	50	200
Scytalidium thermophilum (StL)	0.5	0.4	5
Myceliophthora thermophila (MtL)	0.05	600	1600
Rhus vernicifera (RvL)	0.02	0.05	0.05
Myrothecium bilirubin oxidase (MvBO)	*1.0*	*10*	*10*

*Concentration of NaX (mM) required to reduce the activity of laccase to 1/2 maximum value in 0.1 M Na-acetate, pH 5 with 2 mM ABTS

EPR indicates inhibition occurs at T2 Cu-site

Results imply that access to the T2 Cu-sites varies with source of laccase

Composition of Blood Serum

Arterial Oxygen: 0.21 mM, 0.123 atm partial pressure

Venous Oxygen: 0.09 mM, 0.053 atm partial pressure

Blood Glucose: 3.4-7.2 mM

Blood Serum/Plasma Electrolyte Content

- **Chloride range: 97-107 mM**; Sodium range: 132-144 mM
- Potassium range: 3.6-4.8 mM; Calcium range: 2.0-2.7 mM
- Magnesium range: 0.7-1.2 mM; Bicarbonate range: 9.1-11.7 mM
- Phosphate range: 0.4-0.8 mM; Sulphate range: 0.2-0.3 mM

Minor Blood Serum/Plasma Elements

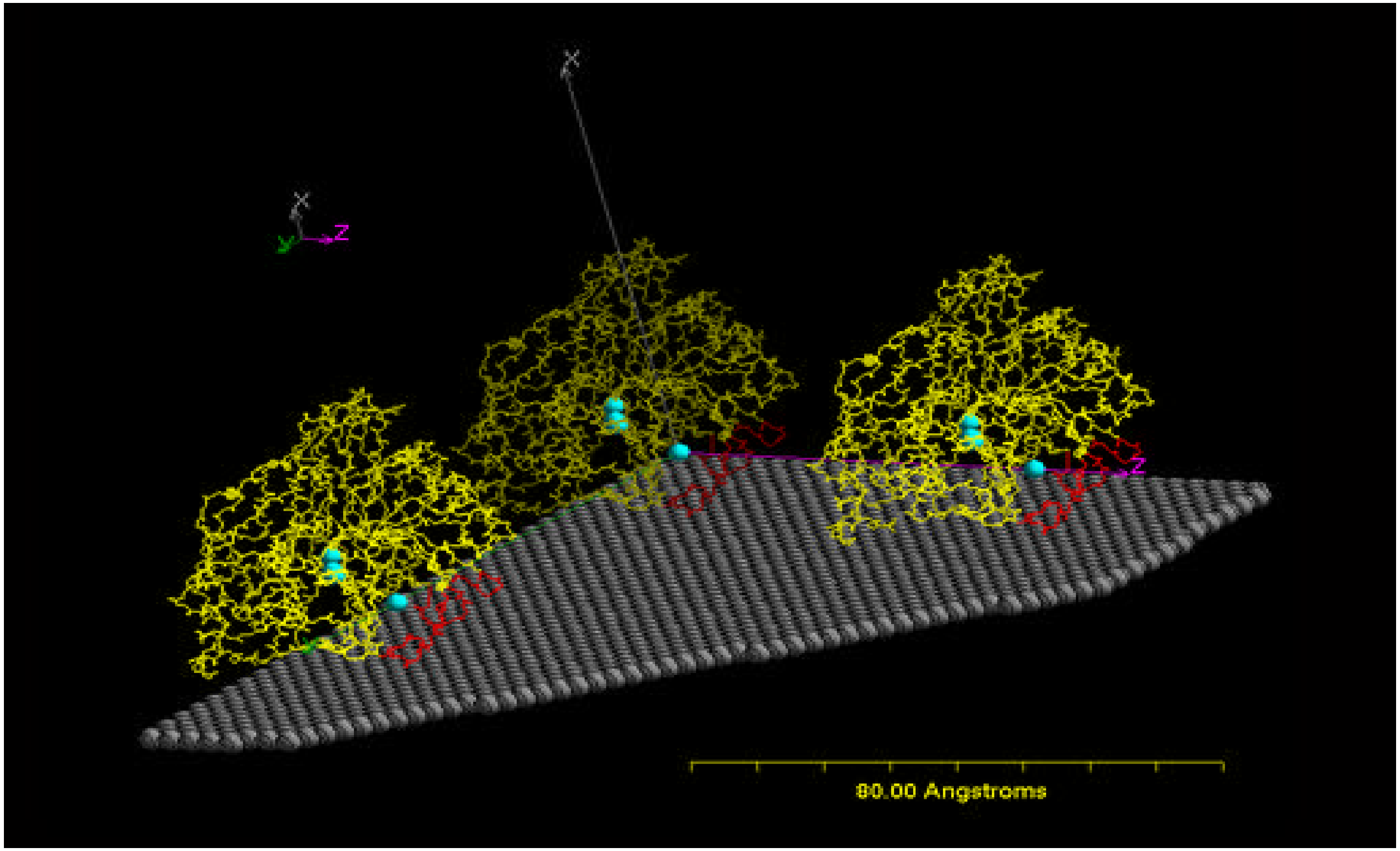
- Aluminum range: 17.0-32.6 uM; **Bromine range: 0.09-0.10 uM**;
- Copper range: 12.0-22.5 uM; **Fluorine range: 5.3-23.7 uM**;
- **Iodine range: 0.4-0.7 uM**; Iron range: 5.7-31.7 uM;
- Lead range: 0.1-0.4 uM; Manganese range: 1.5-3.5 uM;
- Tin range: 0.3-0.8 uM; Zinc range: 0-93.7 uM

Summary of Limitations of Wild-type Laccases

- Specific activity ranges between ~50-500 s^{-1}
- Maximum activity at pH 3.5-4.5
- Activity inhibited by halogen ion (although extent depends on source of laccase)

These limitations may be reduced or eliminated by site-directed mutagenesis or directed molecular evolution (DME)

Altering Structure of Laccase via Site-Directed Mutation of Gene



Site-Directed Mutation of Laccase Gene

Sequence Alignment of T. versicolor Laccases

```
1         10                                            51        60
MGLQRFSFFV TLALVARSLA AIGPVASLVV ANAPVSPDGF LRDAIVVNGV VPSPLITGKK
1                        2  3
GDRFQLNVVD TLTNHSMLKS TSIHWHGFFQ AGTNWADGPA FVNQCPIASG HSFLYDFHVP
1        3  3
DQAGTFWYHS HLSTQYCDGL RGPFVVYDPK DPHASRYDVD NESTVITLTD WYHTAARLGP
1
RFPLGADATL INGLGRSAST PTAALAVINV QHGKRYRFRL VSISCDPNYT FSIDGHNLTV
1
IEVDGINSQP LLVDSIQIFA AQRYSFVLNA NQTVGNYWVR ANPNFGTVGF AGGINSAILR
1
YQGAPVAEPT TTQTTSVIPL IETNLHPLAR MPVPGSPTPG GVDKALNLAF NFNGTNFFIN
1                                                         1 2  3
NATFTPPTVP VLLQILSGAQ TAQDLLPAGS VYPLPAHSTI EITLPATALA PGAPHPFHLH
1                                                      313  1
GHAFAVVRSA GSTTYNYNDP IFRDVVSTGT PAAGDNVTIR FQTDNPGPWF LHCHIDFHLD

1 1                   501        511       519
AGFAIVFAED VADVKAANPV PKAWSDLCPI YDGLSEANQ

AGFAIVFAED VADVKAANPV PKAWSDLCC- ------ ---

AGFAIVFAED VADVKKKNPK PKCCCDCCPI YDGLSEANQ
```

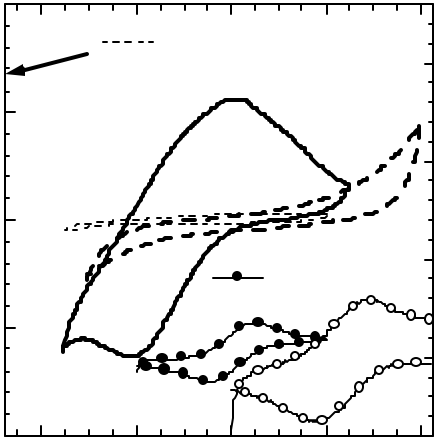
key: signal peptide

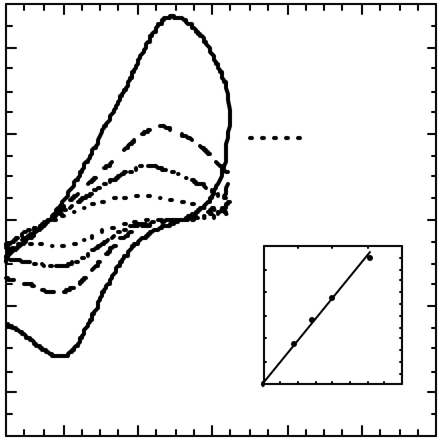
domain 1, domain 2, domain 3

ligands to type **1**, **2**, **3** Cu (I II/II)

mutations

CVs of LccI and LccIa

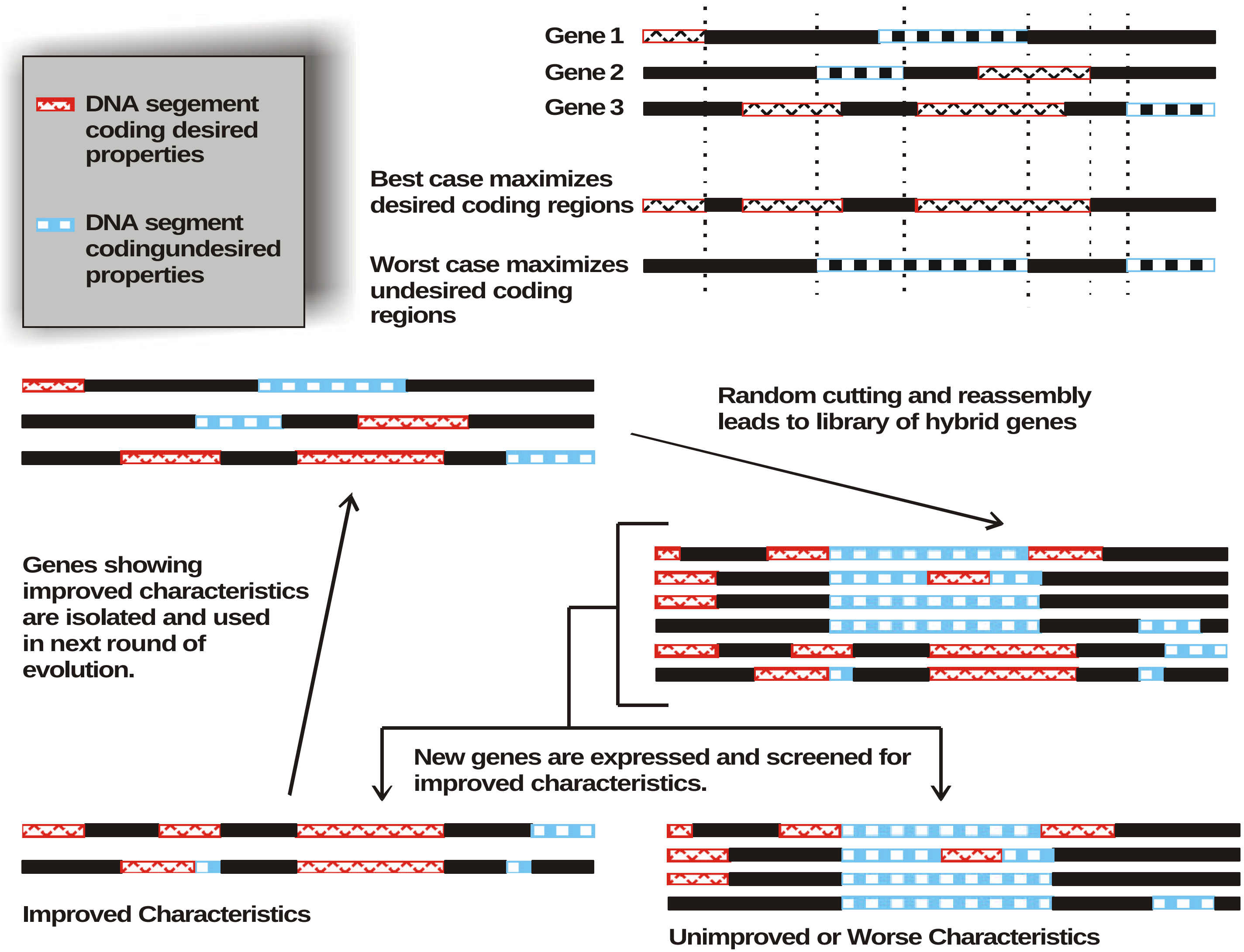




Biochemical changes due to mutation:

- redox potential of T1 Cu-site is more negative
- decreased activity with ABTS
- increased rate of heterogeneous electron transfer

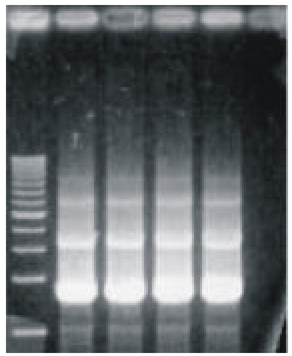
Directed Molecular Evolution of Laccase



Shuffling of Different Laccase Genes

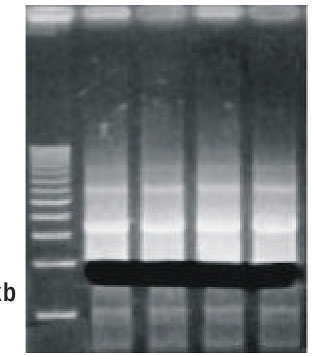
Step 1: Laccase genes isolated from 4 different organisms is replicated using PCR.

1.6kb

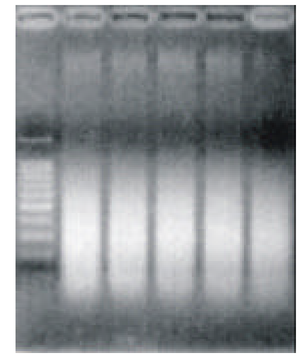


Step 2: The 1.6kb bands of laccase genes are cut from the gel and purified.

1.6kb

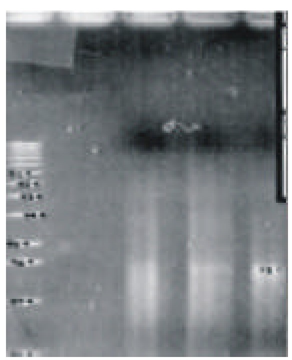


Step 3: The genes are randomly cut by DNAse 1. This causes the gels bands to smear.



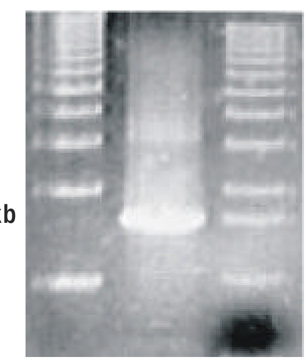
Step 4: Unprimed PCR reassembles 'new' laccase genes..

1.6kb

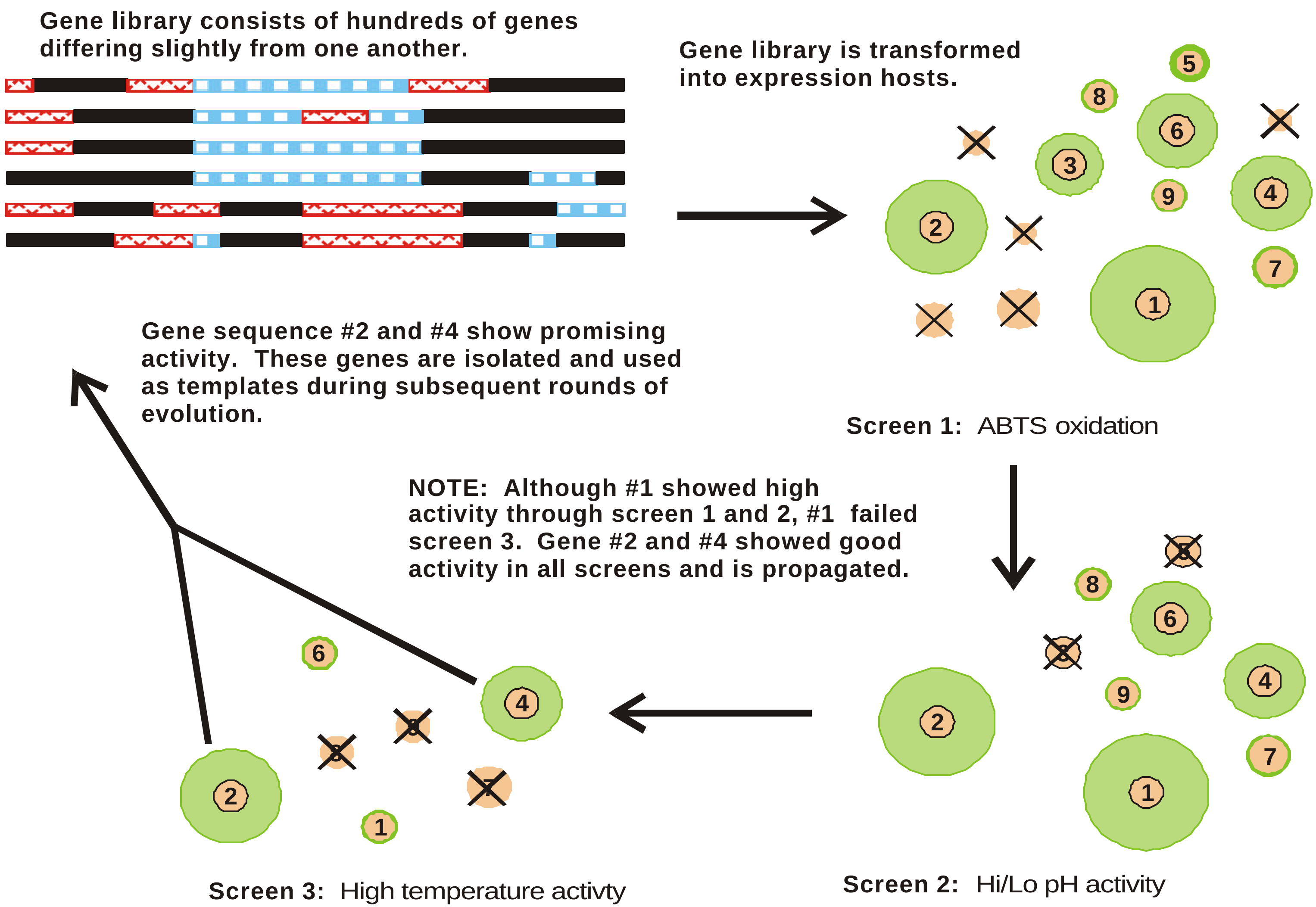


Step 5: Primed amplification of 1.6kb band from step 4.

1.6kb



Screening of Laccase



Laccase Expression in *Pichia Pastoris*

- Yeast strain GS115 transformed with (bottom) and without (top) laccase gene.
- Expression induced with 0.5% methanol.
- ABTS indicator reacts with active laccase to produce green color.

Organisms for Gene Shuffling

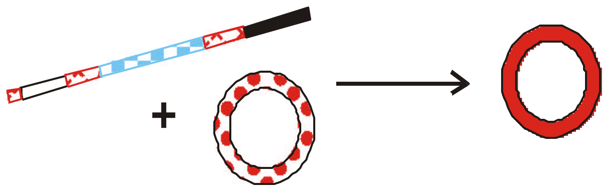
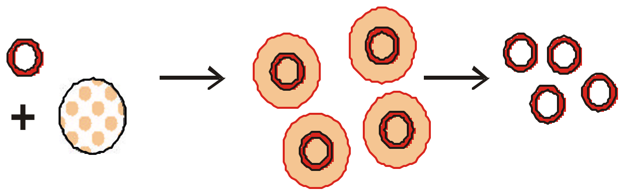
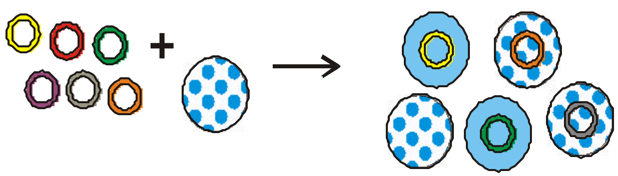
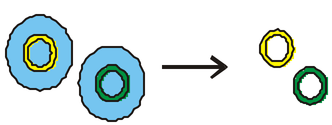
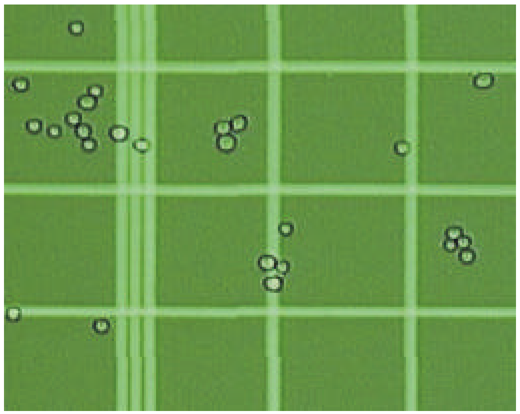
	E. Coli	*S. Cerevisae*	*P. Pastoris*
Formation of Gene Library and Vector Ligation 	24hrs	24hrs	24hrs
Transformation, Replication and Purification of Functional Plasmid from Standard E. Coli. 	24hrs	24hrs	24hrs
Transformation and Growth of Expression Organism 	24hrs	24-60hrs	5-7 days
Screening and Isolation of Genes 	24hrs	24hrs	5-7 days screening 2-3 days Isolation
Total Time per Round of Evolution:	<1 week	<1 week	>2 weeks

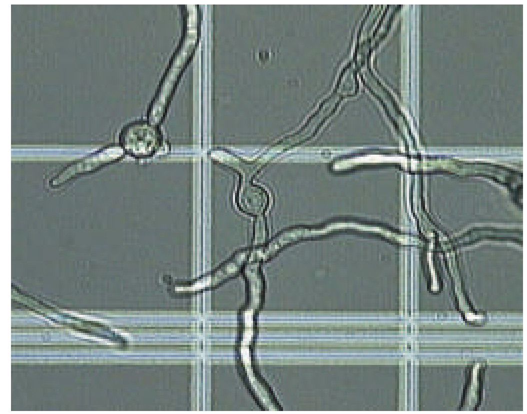
TABLE 2. Yield and activities of LCC I and LCC Ia produced by the SMD1168 strain of *Pichia pastoris*.

	[protein] (mg ml^{-1})	total protein (mg)	total activity (U)	specific activity (mU mg^{-1})
LCC I				
culture (200 ml)	1.6	320	7.82	24
dialysis (10 ml)	4.5	45	7.2	160
post-HPLC (50 ml) dialyzed to 7 ml	0.8	5.6	3.68 6.83	656 (ABTS) 1220 (DEPDA)
LCC Ia				
culture (200 ml)	1.2	240	1.64	7
dialysis (10 ml)	3.1	31	1.52	49
post-HPLC (50 ml) dialyzed to 5 ml	0.5	2.5	0.54	inactive (ABTS) 216 (DEPDA)

A unit of activity (U) is defined as the amount of enzyme that will oxidize 1 x 10^{-6} mol of substrate per minute with the corresponding reduction of dioxygen to water.

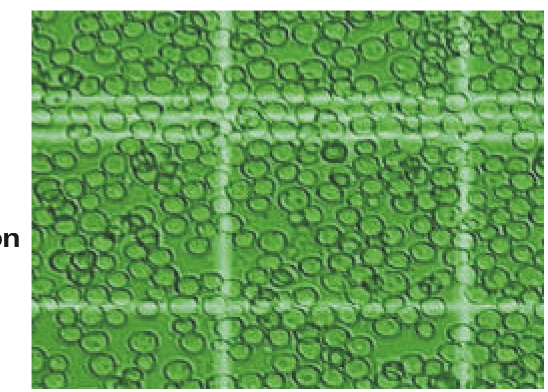
Transformation and Expression of Laccase in *Aspergillus oryzae*



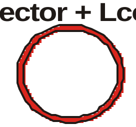


Cell walls are degraded with lysing enzymes for 1-3hrs. Protoplasts result, which are stabilized in an osmotic solution like 1.2M sorbitol.

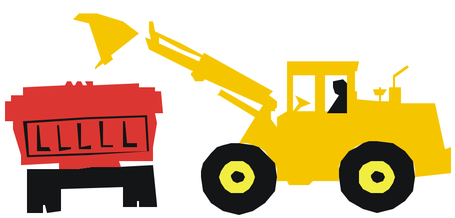
Spores (above) are germinated in growth media overnight (18hrs).



Vector + Lcc



Transformation with glycerol and $CaCl_2$



Fungal proteins like laccase are expressed at very high levels (>10 mg/L) by *Aspergillus oryzae.*

Design of Biofuel Cell

- **Concentration overpotentials**

  Heller fuel cell or alternate design with air-fed cathode?

  limiting current density projected to be ~350 µA cm^{-2} at 0.5 V using $[O_2]_{arterial,\ unbound}$ = 0.21 mM (max. PD ~175 µW cm^{-2})

- **Membrane**

  ion-conduction at pH 7

  biofouling of Nafion

  membraneless biofuel cell?

- **Mediator**

  ABTS-hydrogel to prevent leaching?

  mediatorless electron transfer?

Next Presentation